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Activities of Clarithromycin, Sulfisoxazole, and Rifabutin against *Mycobacterium avium* Complex Multiplication within Human Macrophages

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The activities of clarithromycin, sulfisoxazole, and rifabutin against three virulent strains of *Mycobacterium avium* complex isolated from patients with acquired immunodeficiency syndrome were evaluated in a model of intracellular infection. Human monocyte-derived macrophages were infected at day 6 of culture with *M. avium* complex. Intracellular bacteria were counted 60 min after inoculation. Extra- and intracellular bacteria were counted at days 4 and 7 after inoculation. The concentrations used were 4 μg of clarithromycin per ml (MICs for the three strains, 4, 4, and 4 μg/ml), 50 μg of sulfisoxazole per ml (MICs, 50, 25, and 25 μg/ml), and 0.5 μg of rifabutin per ml (MICs, 2, 0.5, and 0.5 μg/ml). Compared with controls, clarithromycin and rifabutin slowed the intracellular replication of the three strains (at day 7 after inoculation, P was <0.01 for the first strain and <0.001 for the two others). Sulfisoxazole was ineffective against the three strains. Clarithromycin was as effective as rifabutin. Clarithromycin plus rifabutin was as effective as each single agent. Clarithromycin plus sulfisoxazole was as effective as clarithromycin alone.

Mycobacterium avium complex is a primarily intracellular bacterium which multiplies within phagocytic cells. *M. avium* complex infection is a frequent opportunistic infection in patients with acquired immunodeficiency syndrome (AIDS). *M. avium* complex is resistant to most antituberculous agents but may be susceptible in vitro and in vivo to drugs such as amikacin, rifabutin, clofazimine, and fluoroquinolones (2, 6, 7, 10, 14). Combinations of these drugs may control partially human *M. avium* complex infections in patients with AIDS but not achieve complete elimination of the bacteria (8, 21). Thus, the activity of other antimicrobial agents must be tested. Because of the intracellular multiplication of *M. avium* complex, the MIC determination is not sufficient to predict the in vivo efficacy of the agents tested. A cell model was used to assess the activity of antimicrobial agents against *M. avium* complex multiplying within human macrophages (2, 3). Inhibitory activity in vitro of new macrolides such as clarithromycin and of sulfisoxazole against *M. avium* complex has been suggested (2, 5, 12; O. G. W. Berlin, M. N. Clancy, and D. A. Bruckner, Program Abstr. 28th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 1227, 1988). The activities of these two agents were compared in this cell model to that of rifabutin, a rifamycin, which has been tested in a mouse cell model and which is used in clinical trials for the treatment of *M. avium* complex infections (8, 14, 20).

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MATERIALS AND METHODS

Bacteria. Three strains of *M. avium* complex, LA-1, F-2, and D-3, were isolated from patients with AIDS and used after a single culture on Mycobacteria 7H11 agar (Difco Laboratories, Detroit, Mich.) supplemented with Middlebrook OADC enrichment (Difco). One flat, transparent colony of each strain was picked and cultivated at 37°C in Middlebrook 7H9 broth (Difco) supplemented with Middlebrook ADC enrichment (Difco) in Falcon tissue culture flasks (Becton Dickinson Labware, Oxnard, Calif.). After 26 days of culturing, the bacterial suspension was adjusted to a density of 1 mg/ml with a turbidimeter (Institut Pasteur Production). For each strain, counts of CFU on 7H11 agar correlated this density to a bacterial concentration of 2×10^8 CFU/ml. Aliquots of the bacterial suspension were frozen at −80°C.

Antimicrobial agents. The following antimicrobial agents were used: rifabutin (Farmitalia-Carlo Erba, Milan, Italy) and clarithromycin and sulfisoxazole (Abbott Laboratories, North Chicago, Ill.). Stock solutions of each drug were prepared in accordance with manufacturer instructions. From these stock solutions, working solutions were made in distilled water to be incorporated in the media used. The concentration of each drug was close to the peak concentration obtained in the serum of humans given the drug orally (11, 15) (for clarithromycin, Abbott Laboratories, personal communication). These concentrations were equal to or higher than the MICs for the strains of *M. avium* complex, except for strain LA-1, for which the concentration of rifabutin was 1/4× MIC.

Combinations of clarithromycin and rifabutin and of clarithromycin and sulfisoxazole were also tested. The concentrations used for the combinations were the same as those used for the single drugs.

MIC determination by the agar macrodilution method. Serial twofold dilutions of each antimicrobial agent were incorporated into 7H11 agar medium plated in quadrant petri dishes. The inoculum was made from a 7-day-old culture in Dubos-Tween medium and was adjusted to 1 mg/ml (wet weight) and diluted to 10^{-3} and 10^{-5}. From each dilution,

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0.05 ml was plated on one quadrant. Every assay was duplicated. Plates were incubated at 37°C, and colonies were counted after 14 and 21 days of culturing. The lowest concentration of drug that inhibited more than 99% of the bacterial population was considered to be the MIC (16).

Monocyte-derived macrophages. Human monocyte-derived macrophages were obtained from healthy donors. Briefly, 60 ml of peripheral blood was drawn, heparinized, and mixed with 30 ml of RPMI 1640 tissue culture medium (GIBCO Laboratories, Grand Island, N.Y.). This suspension was centrifuged on Ficoll-Hypaque to obtain purified leukocytes. The cells were washed twice in RPMI 1640 medium, and monocytes were counted by the esterase staining method. The cell suspension was distributed into Lab-Tek chambers (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) so as to obtain 5×10^5 monocytes per chamber. After sedimentation for 3 h at 37°C, the supernatant containing nonadherent cells was removed, and adherent cells corresponding to monocytes were incubated in RPMI 1640 medium containing 10% normal human serum in a 5% CO_2 atmosphere. This nutrient medium was changed on days 1 and 3. On day 6, a homogeneous monolayer of macrophages was obtained.

Infection of macrophage monolayers. For the inoculum, an aliquot of the frozen suspension of *M. avium* complex was thawed in warm water and diluted with RPMI 1640 tissue culture medium containing 10% human normal serum so as to obtain a concentration of 3×10^7 CFU/ml. Macrophages were inoculated on day 6 of culture with 1 ml of this *M. avium* complex suspension and incubated for 60 min at 37°C in a 5% CO_2 atmosphere. After ingestion, extracellular bacteria were removed by four washings with phosphate-buffered saline with Ca^{2+} ions (pH 7.2). In control chambers, intracellular bacteria were counted by enumeration of acid-fast bacilli and CFU. In other chambers, fresh RPMI 1640 medium containing 10% normal human serum and one of the antimicrobial agents was added, and macrophages were incubated as described above.

On day 4 after inoculation, extracellular CFU were counted in supernatants. Half of each chamber was used for intracellular acid-fast bacillus and CFU counting; the other half was used to prolong culturing to day 7. In these latter chambers, supernatants were replaced on day 4 by the same fresh medium containing the antimicrobial agent at the same concentration (see above). On day 7 after inoculation, intracellular and extracellular bacteria were counted by the same methods.

The density of the macrophage monolayers was checked at 60 min, day 4, and day 7 with a microscope. The proportions of macrophages that detached from the bottom of the chambers after inoculation were 13% between 60 min and day 4 and 33% between 60 min and day 7.

Acid-fast bacillus counts. For the acid-fast staining, infected macrophages were washed three times with phosphate-buffered saline, fixed with 90% ethanol, and stained by the Kinyoun method. The three strains of *M. avium* complex had a good acid fastness. Macrophages and bacilli were counted microscopically, and the index of intracellular bacilli was quantified at 60 min, day 4, and day 7 by multiplying the percentage of macrophages infected by the mean number of bacilli inside macrophages. Since acid-fast bacillus counts are known to be less precise than CFU counts, acid-fast bacillus counts were determined in only one experiment and were not used in statistical analyses.

CFU counts. Extracellular bacteria were removed by three washings with 2 ml of phosphate-buffered saline, and serial dilutions of this bacterial suspension were plated onto 7H11 agar medium. Intracellular bacteria were recovered by disruption of macrophages by the introduction of 2 ml of distilled water over 30 min and mechanical shaking, and serial dilutions of this bacterial suspension were plated onto 7H11 agar medium. Plates were incubated at 37°C, and colonies were counted after 14 and 21 days of culturing.

Expression of CFU count results. At 60 min after inoculation, only intracellular CFU counts were expressed, corresponding to the intracellular inoculum. In this model, the multiplication of bacteria is mainly intracellular. It has been previously shown that *M. avium* complex does not multiply in RPMI 1640 medium alone and multiplies slowly in RPMI 1640 medium with serum and that the observed extracellular accumulation of *M. avium* complex is macrophage dependent (3). The bacterial load of the macrophages that detached from the chambers remained in the supernatant and was counted as extracellular bacteria. Thus, the total multiplication of bacteria at day 4 was expressed by adding intracellular and extracellular CFU counts on day 4. Since extracellular bacteria were removed from the chambers on day 4 to replace the culture medium, the total multiplication of bacteria at day 7 was expressed by adding intracellular and extracellular CFU counts on day 7 plus extracellular CFU counts on day 4. For each strain and each antimicrobial agent, the results of CFU counts were expressed as the mean of at least three experiments ± the standard deviation.

Statistical analysis. For all strains, results of CFU counts were compared between groups at days 4 and 7 and within each group between days 4 and 7 by a one-way analysis of variance. If the F value was significant, a multiple t test was used to compare the means of each group two by two.

RESULTS

MICs of the antimicrobial agents for the three strains of *M. avium* complex. The MICs of the antimicrobial agents for strains LA-1, F-2, and D-3 of *M. avium* complex were, respectively, 2, 0.5, and 0.5 μg of rifabutin per ml; 4, 4, and 4 μg of clarithromycin per ml; and 50, 25, and 25 μg of sulfisoxazole per ml.

Intracellular acid-fast bacillus counts. Results are shown in Table 1. In control chambers without antimicrobial agent, the increase in intracellular acid-fast bacilli between days 0 and 7 reflected the intracellular growth of *M. avium* complex. This increase was slowed by clarithromycin and rifabutin, but sulfisoxazole was poorly effective. Since this method does not allow discrimination between viable and killed bacteria, CFU counts were preferred for the comparison of efficacy of the various antimicrobial agents.

Efficacy of the various antimicrobial agents evaluated by CFU counts. Results are shown in Fig. 1. The analysis of variance permitted, for each strain, comparisons between groups. Bacillary replication between days 4 and 7 was observed in control groups without antimicrobial agents ($P < 0.01$ for strain LA-1 and $P < 0.02$ for strains F-2 and D-3) and in the sulfisoxazole group ($P < 0.01$ for strains LA-1 and D-3 and $P < 0.05$ for strain F-2). In other antimicrobial agent groups, the CFU counts at days 4 and 7 were not statistically different, reflecting the bacteriostatic effect of the drugs tested. Compared with controls, clarithromycin or rifabutin slowed the intracellular replication of the three strains of *M. avium* complex at day 4 ($P < 0.05$ for strain LA-1, $P < 0.01$ for strain F-2, and $P < 0.001$ for strain D-3) and at day 7 ($P < 0.01$ for strain LA-1 and $P < 0.001$ for strains F-2 and D-3). Compared with controls, sulfisoxazole was only effec-

TABLE 1. Indexes of intracellular acid-fast bacilli at the end of ingestion of *M. avium* complex by macrophages (60 min) and after 4 and 7 days of culturing in the absence (controls) or in the presence of different antimicrobial agents[a]

Drug (concn)	Strain	Time after inoculation of macrophages	% of macrophages infected	Mean no. of bacilli/macrophage	Index of intracellular bacilli
None (controls)	LA-1	60 min	89	1.41	126
		Day 4	99	3.35	332
		Day 7	100	9.26	926
	F-2	60 min	87	4.83	421
		Day 4	100	5.22	522
		Day 7	99	12.12	1,200
	D-3	60 min	92	3.48	321
		Day 4	98	7.29	715
		Day 7	100	12.80	1,280
Rifabutin (0.5)	LA-1	Day 4	91	1.73	158
		Day 7	93	1.94	181
	F-2	Day 4	93	5.72	532
		Day 7	96	4.22	406
	D-3	Day 4	92	6.28	578
		Day 7	98	5.07	497
Clarithromycin (4)	LA-1	Day 4	93	2.22	207
		Day 7	95	2.48	236
	F-2	Day 4	92	5.38	495
		Day 7	98	3.43	337
	D-3	Day 4	100	6.12	612
		Day 7	97	4.89	475
Sulfisoxazole (50)	LA-1	Day 4	99	3.33	310
		Day 7	100	5.20	520
	F-2	Day 4	98	6.16	604
		Day 7	100	6.36	636
	D-3	Day 4	90	4.80	432
		Day 7	100	12.10	1,210

[a] Results are from one experiment for each of the three strains.

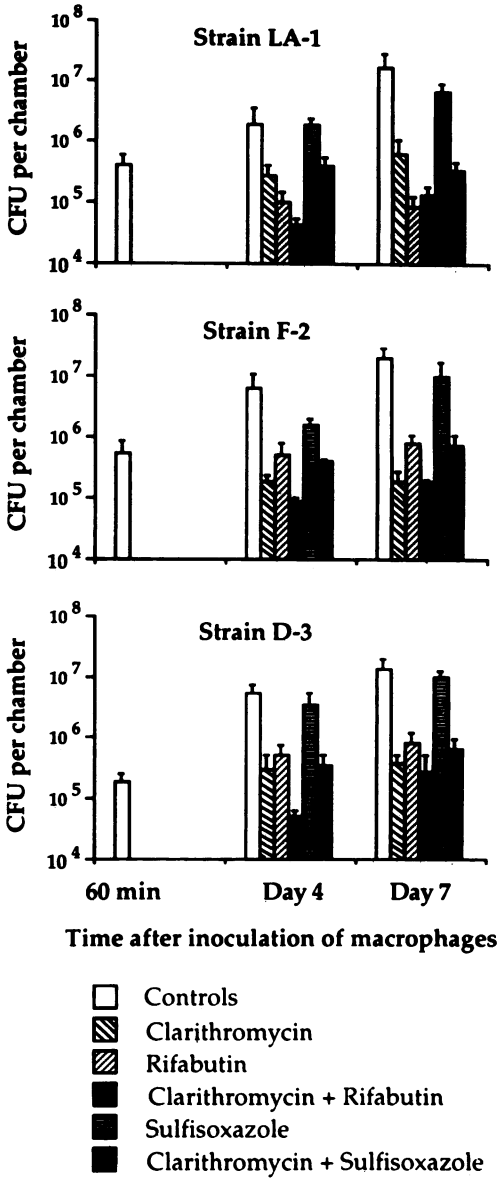

FIG. 1. Comparison of the intracellular growth of three strains of *M. avium* complex in Lab-Tek chambers without antimicrobial agents (controls) and in the presence of different single antimicrobial agents (clarithromycin, 4 μg/ml; rifabutin, 0.5 μg/ml; and sulfisoxazole, 50 μg/ml) or combinations of these agents. The concentrations used for the combinations were the same as those used for the single drugs. Results at day 4 represent intracellular plus extracellular CFU counts on day 4. Results at day 7 represent intracellular plus extracellular CFU counts on day 7 plus extracellular CFU counts on day 4, since these bacteria were removed for medium replacement. Results are the means of at least three experiments ± standard deviations.

tive at day 4 against strain F-2 ($P < 0.05$), was ineffective against the two other strains at day 4, and was ineffective against the three strains at day 7. The efficacy of clarithromycin and that of rifabutin were not statistically different for the three strains at days 4 and 7. The clarithromycin-rifabutin combination was as effective as each single agent at days 4 and 7, and no synergism or antagonism was seen. The efficacy of the clarithromycin-sulfisoxazole combination was not different from that of clarithromycin alone at days 4 and 7.

DISCUSSION

In our cell model, two of the three antimicrobial agents tested, rifabutin and clarithromycin, slowed the intracellular multiplication of *M. avium* complex as compared with controls without antimicrobial agents. The third agent tested, sulfisoxazole, was ineffective against the three strains. Rifabutin is a rifamycin that exhibits in vitro a higher activity than rifampin against *M. avium* complex (4, 9, 16, 18). This activity is mainly bacteriostatic, and a significant bactericidal activity has only been shown with very high concentrations of rifabutin not achievable in the serum of patients or with a prolonged exposure of the bacteria to the drug (13, 19). In a mouse cell model, rifabutin can inhibit the intracellular growth of *M. avium* complex in macrophages (14, 20). Our study showed that similar results could be obtained

in human macrophages. The rifabutin concentration of 0.5 μg/ml used in our experiments was chosen because it is close to the peak concentration obtained in humans after a 600-mg dose given orally (15). In our study, in which the bacteria were inside macrophages, rifabutin was effective against one strain of *M. avium* complex at a concentration of 1/4× MIC, probably because of the intracellular concentration of the drug. It was reported that sulfisoxazole was effective in vitro against *M. avium* complex, with an MIC of 0.2 μg/ml for 90% of the strains tested (Berlin et al., 28th ICAAC). The MIC of sulfisoxazole was much higher for the three strains in our study, between 25 and 50 μg/ml. In our model, sulfisoxazole at an extracellular concentration at least as high as the MIC was ineffective against intracellular *M. avium* complex. Some new macrolides, roxithromycin, azithromycin, and clarithromycin, exhibit in vitro inhibitory activity against *M. avium* complex (12). In two recent studies, the MIC of clarithromycin for 90% of the strains of *M. avium* complex tested was 4 or 8 μg/ml (5, 12). Clarithromycin was also effective against an experimental *M. avium* complex infection of beige mice (5). Our results confirm the good bacteriostatic activity of clarithromycin against intracellular *M. avium* complex and reveal an activity similar to that of rifabutin. The combination of clarithromycin and rifabutin exhibited the same efficacy as did either single drug. The combination of clarithromycin and sulfisoxazole was as effective as clarithromycin alone. In clinical trials for the treatment of *M. avium* complex infections in patients with AIDS, combinations of antimycobacterial agents were often disappointing, since they did not achieve bacterial eradication (1, 8, 17). Thus, new combinations are needed. While sulfisoxazole seems to be of no interest, combinations including clarithromycin should be evaluated in patients with AIDS.

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